Aphidicolin inhibits cell proliferation via the p53-GADD45β pathway in AtT-20 cells

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Abstract. Cushing’s disease is primarily caused by pituitary corticotroph adenomas, which autonomically secrete adrenocorticotropic hormone (ACTH). ACTH production may be associated with tumor cell proliferation; however, the effects of cell cycle progression on ACTH production and cell proliferation are little known in corticotroph tumor cells. A DNA polymerase inhibitor, aphidicolin, arrests cells at the entrance to the S phase and blocks the cell cycle; aphidicolin also induces apoptosis in tumor cells. In the present study, we determined ACTH production and cell proliferation of AtT-20 corticotroph tumor cells following treatment with aphidicolin. Aphidicolin decreased proopiomelanocortin mRNA levels in AtT-20 cells and the levels of ACTH in the culture medium of these cells. Aphidicolin also decreased cell proliferation and induced apoptosis in AtT-20 cells. Fluorescence-activated cell sorting analyses revealed that this agent increased the percentage of G0/G1 phase cells, and decreased S phase cells. Aphidicolin decreased the phosphorylation of cyclic adenosine monophosphate response element-binding protein and Akt. Aphidicolin increased the levels of tumor protein 27 (p27) and 53 (p53), while it decreased cyclin E levels. Aphidicolin also increased the mRNA levels of the stress response gene growth arrest and DNA damage-inducible 45β (GADD45β), a putative downstream target of p53. The p53 knockdown increased GADD45β mRNA levels. The GADD45β knockdown inhibited the decreases in cell proliferation. Thus, aphidicolin inhibits cell proliferation via the p53-GADD45β pathway in AtT-20 cells.

Key words: ACTH, POMC, Pituitary, Cushing’s disease, Stress

CUSHING’S DISEASE is primarily caused by a pituitary adrenocorticotropic hormone (ACTH)-secreting tumor [1, 2]. Excision of the tumor from the pituitary is the primary treatment for Cushing’s disease. However, if excision is unsuccessful, further therapy is needed to treat the resultant hypercortisolism [3]. These therapies include repeat pituitary surgery, radiotherapy, or medical therapy. Some of the drugs used to treat this condition have shown potential therapeutic benefits in limited clinical trials [4]. Drugs that target pituitary ACTH-secreting adenomas include cabergoline and somatostatin analogs, but these are still less popular as standard treatments [4].

Pituitary corticotroph adenomas autonomically secrete ACTH. The precursor of ACTH, proopiomelanocortin (POMC), is cleaved to β-lipotrophic hormone (β-LPH) and ACTH by a processing enzyme, prohormone convertase (PC)-1/3 [5, 6]. β-LPH and ACTH are cleaved to β-endorphin and α-melanocyte-stimulating hormone by PC-2, respectively [5, 6]. Thus, PC-1/3 expression contributes to the production of biologically active ACTH [7, 8]. Additionally, ACTH production may be coordinated with cell proliferation in tumor cells [9]; however, the effects of cell cycle progression on ACTH production and cell proliferation are little known in corticotroph tumor cells. The DNA polymerase inhibitor aphidicolin, arrests cells at the entrance to the S phase and blocks the cell cycle [10]. Cell synchronization is often achieved by inhibition of DNA replication. Aphidicolin also induces apoptosis.
Tumor protein 27Kip1 (p27) and p53 (p53) serve as tumor suppressors. As a critical cell cycle regulator, p27 arrests cell division and inhibits the G1/S transition. Cyclin E forms a complex with cyclin-dependent kinase 2 (CDK2), and cyclin E/CDK2 complexes regulate multiple cellular processes. Cyclin E/CDK2 plays a critical role in the G1 phase and in the G1-S phase transition, and is negatively regulated by p27 [12]. The stress response growth arrest and DNA damage-inducible 45 (GADD45) gene family also participates in cell cycle control, cell survival, apoptosis, maintenance of genomic stability, DNA repair, and active DNA demethylation [13]. GADD45β is a novel pituitary suppressor whose expression blocks proliferation, survival, and tumorigenesis, and it is a putative downstream target of p53 [14].

In the present study, we first examined the effects of aphidicolin on ACTH production and cell proliferation in AtT-20 corticotroph tumor cells. To elucidate further the possible mechanisms of aphidicolin in the pituitary, we then assessed cell cycle profiles and examined the direct effects of aphidicolin on apoptosis and the mRNA levels of GADD45β in AtT-20 corticotroph tumor cells.

### Materials and Methods

#### Materials

Aphidicolin was purchased from Calbiochem (San Diego, CA).

#### Cell culture

AtT-20 pituitary corticotroph tumor cells were cultured in a T75 culture flask with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 U/mL penicillin at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The cells were plated in 6-well plates at 15.0 × 10⁴ cells/cm² for 3 days before each experiment, and the medium was changed every 48 h. To remove the effect of factors contained in FBS, the cells were washed and then starved overnight using DMEM supplemented with 0.2% bovine serum albumin prior to each experiment. At the end of each experiment, total cellular RNA or protein was collected and stored at -80°C until the relevant assay was performed.

#### RNA extraction

The cells were incubated with medium alone (control) or medium containing aphidicolin for the indicated times. To examine the dose-dependent effects of aphidicolin, the cells were incubated for the indicated times with medium alone (control) or medium containing increasing concentrations of aphidicolin (100 nM – 10 µM). At the end of each experiment, total cellular RNA was extracted using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. cDNA was synthesized from total RNA (0.5 µg) using random hexamers as primers with the SuperScript First-Strand Synthesis System for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) (Invitrogen Corp., Carlsbad, CA), according to the manufacturer’s instructions.

#### Quantitative real-time RT-PCR

Total cellular RNA extraction and cDNA synthesis were performed as described previously [15, 16]. The resulting cDNA was then subjected to real-time PCR according to the MIQE guidelines [17]. The expression levels of mouse POMC and GADD45β mRNA were evaluated using quantitative real-time PCR with specific sets of primers and probes (Assays-on-Demand Gene Expression Products; Applied Biosystems, Foster City, CA). β2-Microglobulin (B2MG) was used as a reference gene to standardize expression levels as B2MG mRNA levels did not change during any treatments in this study. Each reaction consisted of 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 1× Assays-on-Demand Gene Expression Products (Mm00435874 m1 for mouse POMC, Mm00435121 g1 for mouse GADD45β, and Mm00437762 m1 for mouse B2MG) and 500 ng cDNA in a total volume of 25 µL with the following parameters on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems): 95°C for 10 min and then 40 cycles at 95°C for 15 s and 60°C for 1 min.

The above assays involved specific sets of primers and a TaqMan probe spanning the exon/exon junction and should not, therefore, have been influenced by DNA contamination. Data were collected and recorded with ABI PRISM 7000 SDS software (Applied Biosystems) and expressed as a function of the threshold cycle (C_T). The amplification efficacies for each gene of interest and the reference gene amplifiers were found to be identical when analyzed with diluted samples.

Relative quantitative gene expression was calcu-
Effects of aphidicolin on AtT-20 tants were measured using an ACTH enzyme-linked immunosorbent assay (ELISA) Kit (MD Bioproducts, Zurich, Switzerland). All samples from each experiment were determined in the same assay.

**Cell proliferation assay**

The cells were incubated at 37°C for 48 h with the indicated concentrations of the agent. Viable cells were measured using a Cell Counting Kit-8 (Dojin, Kumamoto, Japan). All samples from each experiment were determined in the same assay.

**Cell death detection assay**

The cells were incubated at 37°C for 24 h with the indicated concentrations of the agent. DNA fragmentation was measured using a Cell Death Detection ELISA Kit (Roche, Penzberg, Germany), and each enrichment factor was calculated according to the manufacturer’s instructions.

**Cell cycle analysis**

AtT-20 cells were incubated for 24 h with 10 µM aphidicolin or vehicle (dimethyl sulfoxide [DMSO]). Untreated controls were chosen as reference samples, and the ΔC_T for all experimental samples was reduced by the average ΔC_T for the control samples (ΔΔC_T). Finally, the abundance of the experimental mRNA relative to that of the control mRNA was calculated with use of the formula 2^{-ΔΔC_T}.

**Western blot analysis**

Western blot analysis was performed to examine the protein levels of phosphorylated cyclic adenosine monophosphate response element-binding protein (pCREB)/CREB, phosphorylated Akt (pAkt)/Akt, p27, p53, cyclin E, and β-actin (actin). The cells were washed twice with phosphate-buffered saline (PBS) and lysed with Laemmli sample buffer. Cell debris was pelleted by centrifugation, and the supernatant was recovered. The samples (6 µg/each sample) were boiled and subjected to electrophoresis on a 4–20% gradient polyacrylamide gel, and the proteins were transferred to a polyvinylidene fluoride membrane (Daiichi Kagaku, Tokyo, Japan). After blocking with Detector Block® buffer (Kirkegaard & Perry Laboratories, Gaithersburg, MD), the membrane was incubated for 1 h with each antibody (anti-pCREB (dilution 1/500)/CREB (dilution 1/500) antibodies, Cell Signaling Technology, Beverly, MA; anti-pAkt (dilution 1/500)/Akt (dilution 1/2000) antibodies, Cell Signaling Technology; p27 (dilution 1/1000), Santa Cruz Biotechnology, Santa Cruz, CA; p53 (dilution 1/1000), Santa Cruz Biotechnology; cyclin E (dilution 1/250), Santa Cruz Biotechnology; and anti-β-actin (dilution 1/1000) antibody, ab8227 Abcam, Cambridge, MA), washed with PBS containing 0.05% Tween 20, and incubated with horseradish peroxidase-labeled anti-rabbit immunoglobulin G (dilution 1/20000, Daiichi Kagaku). The chemiluminescent substrate SuperSignal West Pico (Pierce Chemical Co., Rockford, IL) was used for detection, and the membrane was exposed to BioMax film (Eastman Kodak Co., Rochester, NY).

**ACTH assay**

The cells were incubated at 37°C for 24 h with the indicated concentrations of the agent. The medium was then aspirated, and ACTH levels in the superna-

**RNA interference experiments**

GADD45β, p53, and control small interfering RNAs (siRNAs) were designed and purchased from QIAGEN. The cells were transfected with siRNA and HiPerFect transfection reagent (QIAGEN) according to the manufacturer’s protocol. For a measurement of mRNA levels, the cells, seeded into 12-well plates at a density of 12 × 10^4 cells/well, were incubated for 24 h in 1 mL of culture medium containing siRNA for either control (siControl) or p53 (sip53, Mm_Trp53_5). The mRNA expression was examined by quantitative RT-PCR. For a measurement of cell proliferation, the cells, seeded into 96-well plates at a density of 1.5 × 10^4 cells/well, were incubated for 24 h in 200 µL of culture medium containing siRNA for either control (siControl) or GADD45β (si GADD45β, Mm_GADD45b_4). The cells were then incubated with vehicle or aphidico-
lin for 48 h. Viable cells were measured using a Cell Counting Kit-8.

Statistical analysis
Each experiment was performed at least 3 times. Samples were provided in triplicate for each group of experiments. Each value is expressed as the mean ± standard error of the mean. Statistical analysis was performed with analysis of variance (ANOVA), followed by Fisher’s protected least-significant difference post hoc test. The level of statistical significance was set at \( P < 0.05 \).

Results

Effects of aphidicolin on POMC mRNA and ACTH levels
AtT-20 cells were incubated with aphidicolin to determine its effects on the time- and dose-dependent changes of POMC mRNA and ACTH levels. A time course study showed that 1 µM aphidicolin significantly decreased POMC mRNA levels (ANOVA; \( P < 0.05 \), Fig. 1A); POMC mRNA levels fell to 60% of the control value within 24 h of adding 1 µM aphidicolin (Fig. 1A). POMC mRNA levels decreased in a dose-dependent manner (ANOVA; \( P < 0.05 \)), with significant effects observed from 1 to 10 µM (Fig. 1A). A time course study showed that 1 µM aphidicolin significantly decreased ACTH levels in the medium within 24 h of the addition (\( P < 0.05 \), Fig. 1B); ACTH levels in the medium also decreased in a dose-dependent manner (ANOVA; \( P < 0.0001 \)), with significant effects observed at 10 µM aphidicolin (Fig. 1B).

Effects of aphidicolin on cell proliferation and cell death
AtT-20 cells were incubated with aphidicolin to determine its effects on the dose-dependent changes in cell proliferation and cell death. Cell proliferation decreased in a dose-dependent manner (ANOVA; \( P < 0.001 \)), with significant effects observed from 1 to 10 µM (Fig. 2A). To examine whether aphidicolin induced apoptosis, cytoplasmic histone-associated DNA fragmentation was determined. Aphidicolin significantly increased DNA fragmentation in a dose-dependent manner (ANOVA; \( P < 0.0001 \)), with significant effects observed at 1 and 10 µM aphidicolin (Fig. 2B).

Effects of aphidicolin on cell cycle profiles
Cell cycle distribution was assessed using flow cytometry. Total cell counts were not changed after incubation with aphidicolin. FACS analyses revealed that the percentage of cells in the G0/G1 phase significantly increased by incubation for 24 h with 10 µM aphidicolin, compared with the control, whereas the percentage of cells in the G2/M phase was not changed, and the percentage of cells in the S phase decreased after incubation (Fig. 3).

Time-dependent changes in aphidicolin-induced CREB and Akt phosphorylation
AtT-20 cells were incubated with 10 µM aphidicolin to determine its effects on CREB and Akt phosphorylation. A time-course study showed that aphidicolin significantly decreased CREB phosphorylation from 30 to 360 min, and Akt phosphorylation from 30 to 120 min in AtT-20 cells (ANOVA; \( P < 0.05 \) and \( P < 0.01 \), respectively; Fig. 4).

Time-dependent changes in aphidicolin-induced p27, p53, and cyclin E protein expression
Aphidicolin (10 µM) significantly increased p27 protein levels from 30 min to 6 h, and p53 protein levels from 2 to 24 h in AtT-20 cells, while it decreased cyclin E levels from 30 min to 24 h (Fig. 5).

Effects of aphidicolin on GADD45β mRNA levels
AtT-20 cells were incubated with aphidicolin to determine its effects on the time- and dose-dependent changes in GADD45β mRNA levels. A time-course study also showed that 10 µM aphidicolin significantly increased GADD45β mRNA levels (ANOVA; \( P < 0.0001 \); Fig. 6). The maximum effect of aphidicolin was observed at 24 h, with an approximately 5.7-fold increase in GADD45β mRNA levels compared with the basal level. Aphidicolin significantly stimulated GADD45β mRNA levels in a dose-dependent manner (ANOVA; \( P < 0.0001 \)), with significant effects observed at 1 and 10 µM (Fig. 6).

Effects of p53 on GADD45β mRNA levels
The functional role of p53 was also examined in AtT-20 cells, since p53 might be involved in the regulation of cell proliferation. The p53 mRNA levels were reduced by 43% in cells transfected with siRNA against the gene. The p53 knockdown significantly increased GADD45β mRNA levels (\( P < 0.05 \)) (Fig. 7).

Effects of GADD45β on cell proliferation
We then examined the functional role of GADD45β
Fig. 1  Effects of aphidicolin on POMC mRNA and ACTH levels in AtT-20 cells. Control cells were treated with medium alone. The cells were treated in triplicate, with the average of three independent experiments (n = 3) shown. Statistical analysis was performed using one-way ANOVA, followed by a Fisher’s protected least-significant difference post hoc test. *P < 0.05 (compared with control [C]). (A) Time-dependent effects of aphidicolin on POMC mRNA levels (left panel): the cells were incubated with medium containing 1 µM aphidicolin. Dose-dependent effects of aphidicolin on POMC mRNA levels (right panel): the cells were incubated for 24 h with medium containing from 100 nM to 10 µM aphidicolin. (B) Time-dependent effects of aphidicolin on ACTH levels (left panel): the cells were incubated with medium containing 1 µM aphidicolin [A]. Dose-dependent effects of aphidicolin on ACTH levels (right panel): the cells were incubated for 24 h with medium containing from 100 nM to 10 µM aphidicolin.

Fig. 2  Effects of aphidicolin on cell proliferation and cell death in AtT-20 cells. Control cells were treated with medium alone. The cells were treated in triplicate, with the average of three independent experiments (n = 3) shown. Statistical analysis was performed using one-way ANOVA, followed by a Fisher’s protected least-significant difference post hoc test. *P < 0.05 (compared with control [C]). (A) Dose-dependent effects of aphidicolin on cell proliferation: the cells were incubated for 48 h with medium containing from 100 nM to 10 µM aphidicolin. Viable cells were measured using a Cell Counting Kit-8. (B) Dose-dependent effects of aphidicolin on cell death: the cells were incubated for 24 h with medium containing from 100 nM to 10 µM aphidicolin. DNA fragmentation was measured using a Cell Death Detection ELISA Kit.
Fig. 3 Effects of aphidicolin on cell cycle profiles in AtT-20 cells. Control cells were treated with medium alone. A representative blot is shown in (A). The cells were treated in duplicate, with the average of three independent experiments (n = 3) shown in (B). Statistical analysis was performed using one-way ANOVA, followed by a Fisher’s protected least-significant difference *post hoc* test. *P* < 0.05 (compared with control). The cells were incubated for 24 h with 10 µM aphidicolin [A] or vehicle (DMSO; control [C]). Cellular DNA content was analyzed with FACS analysis, and the cell cycle profiles were determined with BD FACSDiva™ software.

Fig. 4 Time-dependent changes in aphidicolin-induced CREB and Akt phosphorylation in AtT-20 cells. The cells were incubated with medium containing 10 µM aphidicolin for the durations shown. Western blot analysis was performed to examine the protein levels of phosphorylated (p) CREB/CREB and pAkt/Akt. The figure is shown with the average of 3 independent experiments, and a representative blot is also shown. Statistical analysis was performed using one-way ANOVA, followed by a Fisher’s protected least-significant difference *post hoc* test. *P* < 0.05 compared with control.
in AtT-20 cells, since GADD45β might be involved in the regulation of cell proliferation. GADD45β mRNA levels were reduced by 58% in cells transfected with siRNA against the GADD45β gene. GADD45β knockdown did not modify the basal cell proliferation (Fig. 8). Aphidicolin significantly decreased cell proliferation. GADD45β knockdown significantly inhibited the decreases in cell proliferation ($P < 0.05$) (Fig. 8).

**Discussion**

In this study, we found that aphidicolin decreased POMC mRNA levels in AtT-20 cells and the levels of ACTH in the culture medium of these cells. These results indicate that aphidicolin, a DNA polymerase inhibitor, suppressed the synthesis and secretion of ACTH in corticotroph tumor cells. Our data further suggest that aphidicolin decreased cell proliferation and increased DNA fragmentation in AtT-20 cells. Aphidicolin also induces apoptosis in human cervical cancer HeLa S3 cells [11]. Total ACTH production depends on synthesis and cell proliferation; therefore, decreases in cell growth via apoptosis may contribute to the decreased ACTH levels observed in the culture medium.

POMC mRNA levels were decreased by 1 µM aphidicolin, while ACTH levels in the medium were done by 10 µM aphidicolin within 24 h. The difference in kinetics between POMC mRNA and ACTH levels was found in this study. The expression levels of mRNA are determined mainly by both transcription, such as a synthet-

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**Fig. 5** Time-dependent changes in aphidicolin-induced p27, p53, and cyclin E protein expression in AtT-20 cells. The cells were incubated with medium containing 10 µM aphidicolin for the durations shown. Western blot analysis was performed to examine the protein levels of p27, p53, cyclin E, and β-actin (Actin, control). The figure is shown with the average of 3 independent experiments, and a representative blot is also shown. Statistical analysis was performed using one-way ANOVA, followed by a Fisher’s protected least-significant difference post hoc test. *$P < 0.05$ compared with control.

**Fig. 6** Effects of aphidicolin on GADD45β mRNA levels in AtT-20 cells. Control cells were treated with medium alone. The cells were treated in triplicate, with the average of three independent experiments ($n = 3$) shown. Statistical analysis was performed using one-way ANOVA, followed by a Fisher’s protected least-significant difference post hoc test. *$P < 0.05$ (compared with control [C]). Time-dependent effects of aphidicolin on GADD45β mRNA levels (left panel): the cells were incubated with medium containing 10 µM aphidicolin. Dose-dependent effects of aphidicolin on GADD45β mRNA levels (right panel): the cells were incubated for 24 h with medium containing from 100 nM to 10 µM aphidicolin.
cellular Ca^{2+}, thereby promoting ACTH secretion [20]. In AtT-20 cells, but not anterior pituitary cells, the PKC pathway is also involved in POMC gene expression [21]. Therefore, in AtT-20 cells, the PKA and PKC pathways might participate in gene regulation via CREB phosphorylation. In this study, aphidicolin also decreased Akt phosphorylation. The phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway is an intracellular signaling pathway that contributes to apoptosis and hence cancer. This pathway is overactive, thus reducing apoptosis and allowing proliferation, in a variety of cancer cells. In fact, Akt expression levels are up-regulated in pituitary tumors, including corticotroph tumor cells, compared to normal pituitary cells [12, 22]. Therefore, the Akt pathway may be involved in cell proliferation in the pituitary.

Aphidicolin increased p27 and p53 levels, while it decreased cyclin E levels. Both p27 and p53 are tumor-suppressing proteins that regulate cyclin-dependent proteins, resulting in suppression of the cell cycle. Aphidicolin is known to arrest cells at the entrance to the S phase, thereby blocking the cell cycle [10]. In fact, FACS analyses revealed that this agent decreased...
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Aphidicolin decreased POMC mRNA levels in AtT-20 cells and ACTH levels in the culture medium of these cells. Aphidicolin decreased cell proliferation and induced apoptosis in AtT-20 cells. FACS analyses revealed that this agent decreased the percentage of S phase cells and increased the percentage of G0/G1 phase cells in AtT-20 cells. Therefore, aphidicolin inhibits cell proliferation via the p53-GADD45β pathway in AtT-20 cells.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

Conclusion

In conclusion, the present study demonstrated that aphidicolin decreased POMC mRNA levels in AtT-20 cells and ACTH levels in the culture medium of these cells. Aphidicolin decreased cell proliferation and induced apoptosis in AtT-20 cells. FACS analyses revealed that this agent decreased the percentage of S phase cells and increased the percentage of G0/G1 phase cells in AtT-20 cells. Therefore, aphidicolin inhibits cell proliferation via the p53-GADD45β pathway in AtT-20 cells.

References


